EXHIBIT E

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Early embryonic angiogenesis in the chick area vasculosa

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ABSTRACT

The rate and pattern of growth as well as vessel ultrastructure of the area vasculosa were examined in the chick. The embryos were grown in shell-less culture after 3 d in ovo and staged according to Hamburger & Hamilton (1951) and the rate of increase in the diameter of the area vasculosa was measured. This revealed an increase in the area vasculosa diameter of 0.4 ± 0.02 mm h^{-1} ($n = 62$) for embryos between stages 15 and 20. To determine the growth pattern of the sinus terminalis (the advancing edge of the area vasculosa), a marked length of the sinus was photographed at hourly intervals over a period of 9 h. It was found that this vessel grows by new vessels forming external to the sinus in the form of parallel plexuses, one of which then replaces the original sinus as the major route of bloodflow. Ultrastructurally the capillaries of the area vasculosa were simple tubes of endothelial cells, lacking a basement membrane. The endothelial cell cytoplasm contained only a few organelles, mainly mitochondria and rough endoplasmic reticulum. These findings indicate that the chick area vasculosa capillaries bear similar structural and growth characteristics to those associated with tumour angiogenesis and suggest that they may prove to be a useful model system for studying the factors involved in pathological angiogenesis.

INTRODUCTION

Angiogenesis is the process by which new blood capillaries are formed by sprouting from existing capillaries (Klagsbrun & D'Amore, 1991; Ribatti et al. 1991) as opposed to the earliest blood vessel formation from angioblasts, a process called vasculogenesis (Poole & Coffin, 1989). Angiogenesis is of great importance in embryogenesis (Risau, 1991; Breier et al. 1992) and to a lesser extent in postnatal tissue growth. It is rare in mature tissue, with the exception of the female reproductive system and wound healing. It is, however, also involved in a wide range of pathological conditions termed 'angiogenic diseases', conditions characterised by pathological growth of new capillaries, the most notable of which is solid tumour growth (Moses & Langer, 1991). The process of angiogenesis has been divided into a number of stages (Fraser & Simpson, 1983). These include the migration and proliferation of endothelial cells, with migration being one of the first steps and perhaps the most important as angiogenesis has been demonstrated in the absence of endothelial proliferation (Sholley et al. 1984).

A range of angiogenic factors has been described using a variety of assay systems, most recently based on the early chick extraembryonic membranes (Stewart et al. 1990; Auerbach et al. 1991). Both inhibitory and stimulatory factors have been discovered, and vary in nature from peptide growth factors to fungal antibiotics (Klagsbrun & d'Amore, 1991; Moses & Lange 1991; Oikawa et al. 1991*a*, *b*). The most commonly used angiogenic model is the 8- to 10-d-old chick chorioallantoic membrane (CAM) (Folkman, 1974; Fraser et al. 1983; Auerbach et al. 1991; Pasi et al. 1991), although a second model being increasingly used is the earlier formed yolk-sac membrane of the chick, the area vasculosa (Ausprunk et al. 1974; Defouw et al. 1989; Stewart et al. 1990; Dugan et al. 1991; Otsuka et al. 1992). The yolk-sac (or vitelline) membrane, referred to as the VIM, is composed of 2 distinct areas: the area vasculosa and the nonvascular area vitellina. The area vasculosa is composed of radially growing vessels with lateral connecting vessels, the outer edge of which is marked by a vessel called the sinus terminalis. The area vasculosa spreads over the yolk sac until it almost completely covers it by d 14–15 of incubation. This

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membrane then persists, absorbing yolk nutrients and manufacturing blood until it is retracted into the abdominal cavity on d 20 of incubation (Romanoff, 1960).

The VIM has important advantages over the CAM assay. The extent of the development of the chick immune system at the time when the CAM assay is carried out (8–10 d), may give rise to problems of false positive results due to an inflammatory response evoked by the compounds assayed, or by the delivery medium used (Jakob et al. 1978; Auerbach et al. 1991). These problems are overcome by the VIM assay, as at 3–5 d of chick development, the immune system has not developed. The VIM assay can also be carried out in shell-less culture giving easy access to the whole membrane (Stewart et al. 1990). In view of the increasing use of the VIM as a bioassay, it is necessary to examine the structure and growth pattern of the area vasculosa. This study sets out to examine in detail the nature of the capillaries of the area vasculosa, particularly the extent of the vessel maturation. In addition it has been reported that the growth rate of these capillaries is high with the diameter of the area vasculosa increasing by 7–14 mm per day during stages 16–20 (Romanoff, 1960; Stewart et al. 1990), although it is unclear how this rapid growth is achieved.

MATERIALS AND METHODS

Ross strain chicken eggs were incubated for 3 d at 37.5 °C, and then used to prepare shell-less cultures (Dunn & Boone, 1976; Stewart et al. 1990; Dugan et al. 1991). In brief, the eggs were swabbed with 70% ethanol and under sterile conditions, broken into a shallow well formed by a length of transparent film wrap stretched across the lip of a plastic cup. The resulting cultures were covered with an off-set Petri dish lid to prevent dehydration, whilst still permitting clearance for gas exchange. The cultures were then further incubated at 37.5 °C in a humidified atmosphere.

Embryos between stages 15 and 17 (Hamburger & Hamilton, 1951) were selected for structural and ultrastructural study, and small 20 mm^2 portions of area vasculosa including the sinus terminalis together with adjacent radial vessels and 20 mm^2 portions from the region proximal to the inner area pellucida, including the anterior vitelline vein, were dissected and processed for resin histology. The specimens were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4 °C overnight. After rinsing in 0.1 M cacodylate buffer (pH 7.2), they were then postfixed in 1% osmium tetroxide for 1 h, dehydrated through graded ethanol solutions (50, 70, 90 and 100%) and then transferred to propylene oxide before being embedded in Taab resin. Semithin sections (1 μm) were cut and stained with toluidine blue. From selected areas, ultrathin sections were cut using a Reichert Ultracut E ultramicrotome and stained with uranyl acetate and lead citrate (Reynolds, 1963) for examination by transmission electron microscopy. Representative photographs were taken using a JEOL CX100 electron microscope.

Stage 15 embryos (Hamburger & Hamilton, 1951) were selected for gross measurement of the area vasculosa. The diameter of the area vasculosa was measured with dividers using a Wild M37 binocular dissecting microscope, at 2 h intervals over a period of 6 h and then at 24 h. At each measurement the embryos were staged in order to check that they were developing at similar rates to one another (n = 62).

Three embryos (stage 16) were selected and on the vasculature behind the sinus terminalis a drop of India ink was placed to act as a reference point, and the area of the sinus terminalis adjacent to the ink spot photographed using a camera attached to a dissecting microscope, hourly for 9 h.

RESULTS

The area vasculosa showed the 3 layers of the endoderm, mesoderm and ectoderm. The mesoderm was clearly visible due to the presence of numerous small blood vessels. These vessels were immediately subjacent to a thin ectodermal layer. The blood vessels were simple in appearance. In the sections from the outer edge of the area vasculosa the sinus terminalis was clearly visible as the last blood vessel before the nonvascular area vitellina (Fig. 1*a*). In the

Fig. 1. (*a*) Photomicrograph of a semithin section of the sinus terminalis and adjacent radial vessels. Solid arrows, sinus terminalis; hollow arrows, the area vasculosa. × 225. Inset: detailed structure of the area vasculosa, showing endoderm (En), vascular mesoderm (arrow) and ectoderm (arrowhead). × 370. (*b*) Low-power transmission electron micrograph of a capillary from just behind the sinus terminalis showing the simple structure of these vessels. Arrow, perinuclear region of an endothelial cell. Bar 4 μm. (*c*) Low-power transmission electron micrograph of part of the sinus terminalis showing the same simple structure as in (*b*). The lumen (L) is surrounded by thin wall capillary endothelial cells (arrows, perinuclear regions). Adjacent to the capillary are large extracellular spaces (S) in the mesoderm. Bar, 5 μm. (*d*) Micrograph showing paucity of organelles within the endothelial cell cytoplasm from the sinus terminalis. Mitochondria and some endoplasmic reticulum are present; few other organelles are evident. Arrows, abluminal surface. Bar, 1 μm.

1a
En
b
c
S
L
d

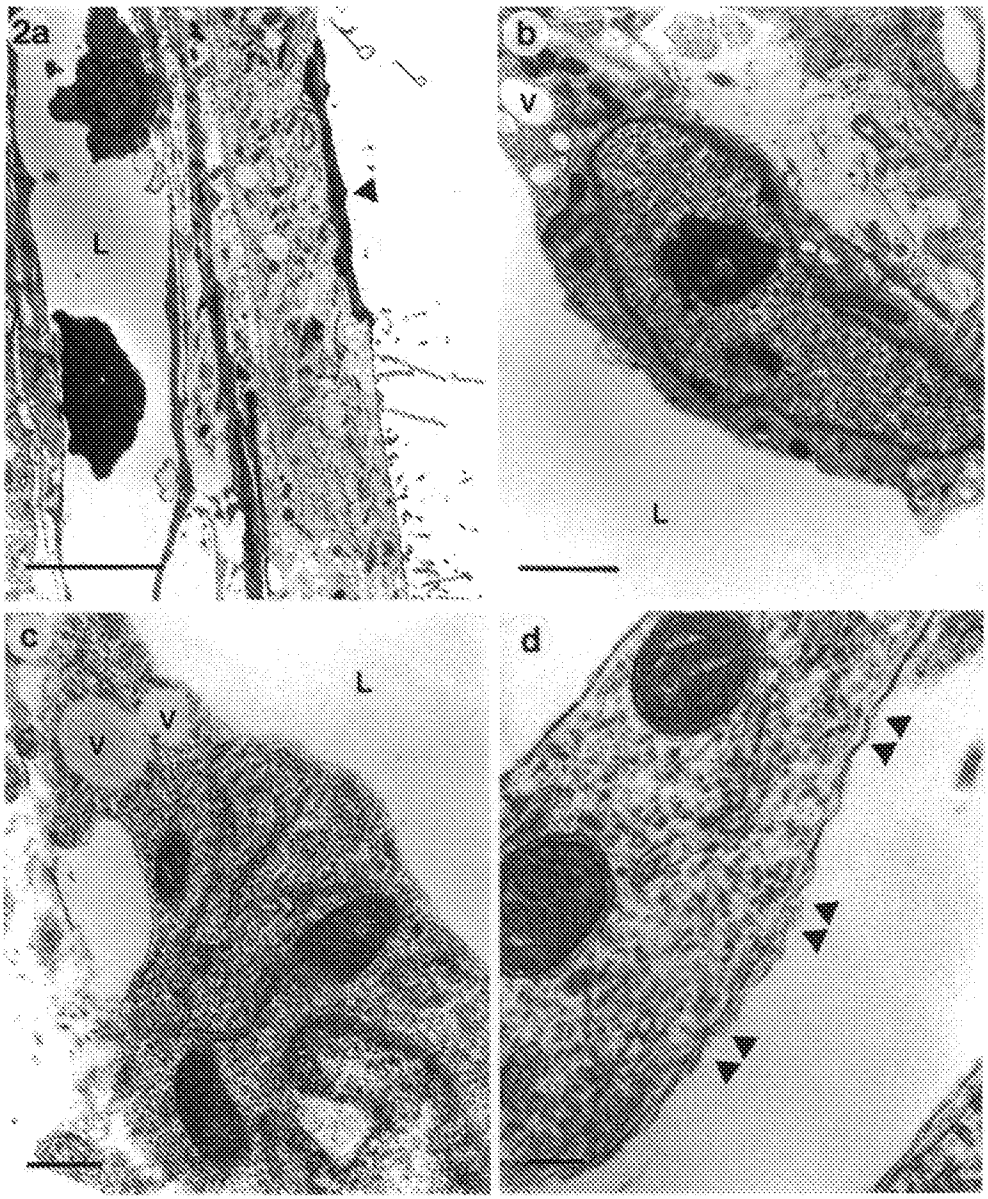

Fig. 2. (*a*) Low-power electron micrograph of small capillary from inner area vasculosa adjacent to anterior part of the embryo showing the same simple structure as those from the outer area vasculosa. L, vessel lumen; arrow head, ectodermal surface. Bar, 5 μm. (*b*) Electron micrograph of perinuclear area of an endothelial cell from inner area vasculosa showing a number of cytoplasmic vacuoles (V); L, vessel lumen. Bar, 2 μm. (*c*) Higher-power micrograph of endothelial cytoplasm from inner area vasculosa showing the typical organelle complement of mitochondria, rough endoplasmic reticulum and vacuoles (V). Bar, 0.5 μm. (*d*) Micrograph showing absence of a basement membrane from capillaries of inner area vasculosa, (arrowheads, abluminal aspect of the cell). Bar, 0.1 μm.

sections from the inner area vasculosa the anterior vitelline vein was clearly visible as the largest vessel with numerous adjacent small capillaries. Ultrastructurally the area vasculosa capillaries were of very simple construction (Figs 1*b*, *c*, 2*a*), the walls of the vessels comprised endothelial cells only, there being no evidence of either apposing pericytes or of an organised basement membrane on the abluminal side of the capillaries in the sinus terminalis, anterior vitelline vein and the radial vessels immediately behind the sinus and adjacent to the anterior vitelline vein. Occasionally extracellular matrix was present between the abluminal side of the endothelial cells and the surrounding mesenchyme (Fig. 1*d*). However, there was no evidence of an organised basement membrane present in these areas. Higher magnifications revealed that the endothelial cells were relatively undifferentiated, the only prominent organelles being the nucleus, mitochondria, rough endoplasmic reticulum and free ribosomes (Figs 1*e*, 2*c*). Endothelial cells adjacent to the anterior vitelline vein exhibited greater numbers of these organelles, and also contained

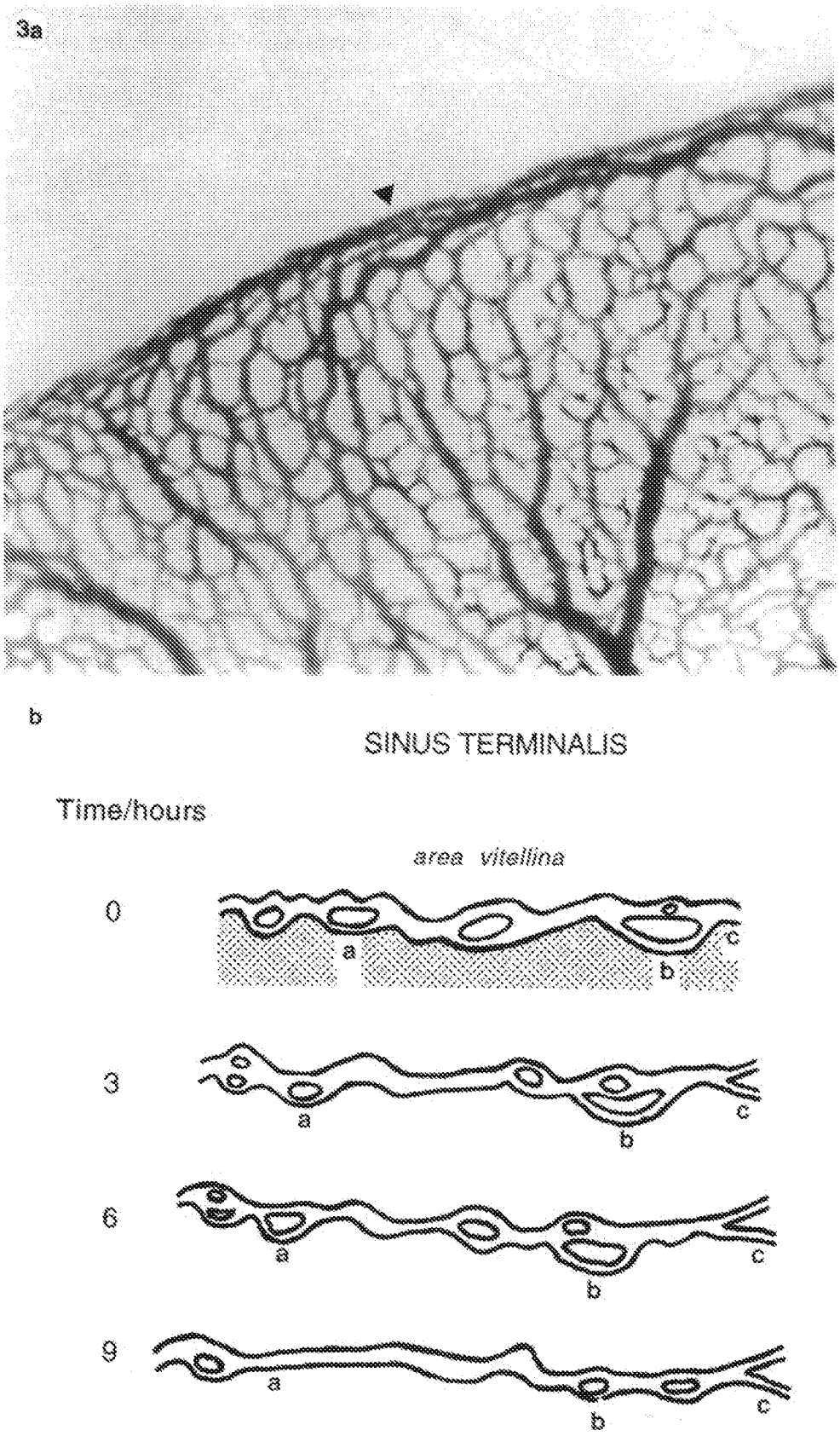

Fig. 3. (*a*) Micrograph showing area vasculosa including the sinus terminalis. Monastral Blue has been injected to increase the contrast for photography. Numerous plexuses are visible. Arrowhead, sinus terminalis. × 28. (*b*) Diagram illustrating the appearance of a marked stretch of sinus terminalis at 3 h intervals over 9 h. Stippled area, area vasculosa behind the sinus terminalis. Positions *a*, *b* and *c* relate points along the sinus terminalis where changes in the pattern of the plexuses occur over the time period. At 0 h the initial length of the marked sinus terminalis was 5 mm; after 9 h this had increased to 6 mm.

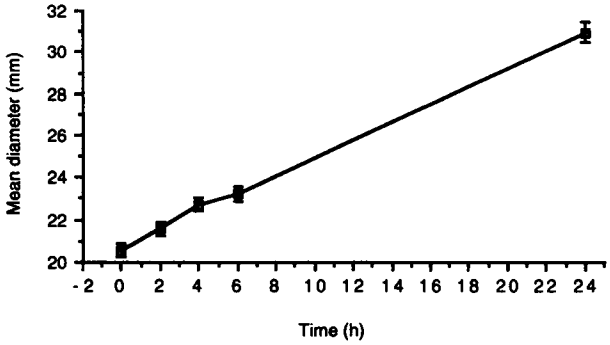

Fig. 4. Graph showing average diameter of area vasculosa at 2 h intervals measured for 6 h, and then measured again after 24 h (±S.E.). The graph indicates that the growth of the area vasculosa is linear (0.4 mm h^{-1}) during the period over which measurements were made (from stages 15 to stages 19–20).

cytoplasmic vacuoles. Several important features of mature endothelial cells were absent, namely micropinocytotic vesicles, Weibel-Palade bodies (Wagner, 1980; Ryan & Barnhill, 1983), obvious cytoskeleton components and junctioning of the cells. For the greater part of the cell length the cytoplasm was very thin, with a thicker bulge containing the nucleus.

A length of the sinus terminalis adjacent to the India ink reference point examined over a period of 9 h revealed that the sinus terminalis was not in fact a single continuous vessel, as has been described, but was in many areas a plexus of parallel vessels (Fig. 3*a*). Comparisons of the stages examined indicated that the plexuses underwent remodelling; thus segments of the single sinus were replaced by a plexus of vessels, one of which eventually became the major bloodflow route, replacing the original segment of the sinus (Fig. 3*b*).

In all, the diameter of the area vasculosa in 62 shell-less cultures was measured. The increase obtained was constant showing linear growth with a mean increase in the diameter of 0.4 ± 0.02 mm h^{-1} (10.4 mm d^{-1}) (Fig. 4).

DISCUSSION

The results of this study show that the area vasculosa capillaries are structurally simple, merely tubes of endothelial cells, with no evidence of apposing pericytes or indeed of a basement membrane. Extracellular matrix between the endothelial cells and the surrounding mesenchyme was not organised into a basement membrane either in the outer or inner regions of the area vasculosa. The presence of a basement membrane in the vessels of the developing area vasculosa has been controversial. Gonzalez-Crussi (1971) noted that there was a complete lack of basement membrane in the area vasculosa capillaries between stages 15 and 22. Basement membrane was noted around some larger vessels, but not at all stages or in all specimens. More recently, Murphy & Carlson (1978) reported a basement membrane being present around all vessels at stage 15, becoming more complete in later stages up to stage 23. Our findings suggest that between stages 15 and 17 the capillaries of the area vasculosa are devoid of a distinct basement membrane. The results also reveal that the endothelial cells of the area vasculosa differ in ultrastructure from mature capillary endothelial cells, having a simple, relatively undifferentiated cytoplasm containing only mitochondria and rough endoplasmic reticulum, suggesting that the cells may be undergoing frequent division rather than specialised endothelial functions (Hudlicka & Tyler, 1986). By contrast, mature capillary endothelial cells have smaller nuclei, fewer mitochondria and numerous cytoplasmic vesicles, indicative of cell transport rather than division (Ausprunk et al. 1974; Wagner, 1980; Hudlicka et al. 1986; Shumko et al. 1988). Cytoplasmic vacuoles were often observed in the endothelial cells in the inner region of the area vasculosa (adjacent to the embryo) but infrequently in the region of the sinus terminalis, suggesting that these cells may exhibit a gradient of heterogeneity reflecting degrees of cytodifferentiation similar to that seen in the developing embryonic limb bud (Wilson & Orr-Urtereger, 1986; Feinberg et al. 1991).

The measurements of the growth rate of the area vasculosa confirm previously reported data (Romanoff, 1960; Stewart et al. 1990), suggesting that the diameter of the area vasculosa increased by between 7 and 14 mm d^{-1}, much faster than that seen in capillaries from mature tissue which grow very slowly or not at all. The rate of growth of area vasculosa capillaries poses the problem of how the circular sinus terminalis could grow to match the rapid radial expansion. Our results show that the sinus terminalis produces angiogenic sprouts which arc from the sinus and rejoin it at another point, forming a plexus. The new vessel length provides an alternative direction for bloodflow and effectively lengthens the sinus terminalis.

The structure of the area vasculosa capillaries resembles that of tumour capillaries which lack pericytes and have very little basement membrane material (Blood & Zetter, 1990; Paku & Paweletz, 1991). Tumour endothelial cells also have simple cytoplasm with numerous mitochondria and large nuclei (Schor & Schor, 1983). In addition, tumour capillaries have been reported as growing 0.12 to

0.3 mm d^{-1} (Folkman et al. 1983; Schor & Schor, 1983). In these respects the capillaries and endothelial cells of the area vasculosa may be used as a model of capillaries associated with angiogenic diseases (such as tumour-induced capillary growth). This, in conjunction with the advantages that the VIM assay has over other assay systems, makes it a useful model for the development of therapeutic approaches to angiogenic diseases, particularly for assessing the effects of putative angiogenic inhibitors and promoters.

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